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(54) Title: AN ISOLATED NUCLEOTIDE SEQUENCE RESPONSIBLE FOR THE TOMATO DARK GREEN (DG) MUTATION AND USES THEREOF

(57) Abstract: The present invention describes an isolated nucleotide sequence responsible for the tomato dark green (dg) phenotype, wherein said sequence comprises an altered tomato DET1 gene sequence or fragment thereof. Further described is a method for detecting the presence of the dg mutation in a plant, comprising the steps of isolating the genomic DNA from said plant, amplifying a gene fragment containing said dg mutation from said genomic DNA by use of a PCR technique and determining the presence of said dg mutation in said genomic DNA. Further described is a method for preparing double-mutant lines of Lycopersicon esculentum having genotype dg/dg p/p, wherein p represents any recessive photomorphogenic mutation that is genetically unlinked to the dg mutation.

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An Isolated Nucleotide Sequence Responsible for the Tomato *Dark Green* (*dg*) Mutation and Uses thereof

Field of the Invention

The present invention relates to a modified nucleotide sequence that is responsible for producing the *dark green* (*dg*) phenotype in tomatoes. More specifically, the present invention discloses a point mutation in the tomato homolog of the *Arabidopsis DET1* gene and the uses of said modified nucleotide sequence.

Background of the Invention

Plants respond to light intensity, direction, duration, and spectral quality by modulating their developmental processes in an array of interactions that are referred to as photomorphogenesis. Photomorphogenic mutants have been proven to be an excellent tool in research of the complex interactions between light and plant development and some of them have also been used in several agricultural crop breeding programs. Photomorphogenic mutants have been reported in a number of species, including *Arabidopsis*, *Sorghum*, *Brassica*, tobacco, tomato and pea. In general, these mutants may be classified either as defective in photoreceptors, or altered in some aspect of light signal transduction chain (Chory, 1993).

Several light-hypersensitive mutants have been described in tomato (*Lycopersicon esculentum*). Among these, mutants carrying the monogenic recessive *high pigment* (*hp-1* and *hp-2*) and *dark green* (*dg*) mutations are characterized by their exaggerated light responsiveness. These mutants display higher anthocyanin levels, shorter hypocotyls, and greater

fruit pigmentation in comparison to their semi-isogenic wild type plants (Mochizuki and Kamimura 1984; Wann et al. 1985). The increased fruit pigmentation seen in these mutants is due to significantly elevated levels of carotenoids, primarily lycopene, and flavonoids in the mature ripe red fruit. As a consequence of their effect on fruit color, *hp* and *dg* mutations were introgressed into several commercial processing and fresh-market tomato cultivars that are currently marketed as Lycopene Rich Tomatoes (LRT) (Wann, 1997).

The *hp-1* mutant was originally discovered in 1917 as a spontaneous mutant at the Campbell Soup Company farms (Riverton, NJ) (Reynard, 1956), the *hp-2* mutant was reported in the Italian San Marzano variety in 1975 (Soressi, 1975), and the *dg* mutant appeared in trellized planting of the Manapal variety (Konsler, 1973).Despite some initial confusion, it is now clear that *hp-1* and *hp-2* mutations map the tomato chromosomes 2 and 1, respectively, and are therefore non-allelic (Van Tuinen et al. 1997; Yen et al. 1997). At each of these loci, two mutant alleles have been identified: *hp-1* and $hp\text{-}1^{w}$, *hp-2* and $hp\text{-}2^{j}$ (Kerckhoff and Kendrick 1997; Van Tuinen et al. 1997).

WO 99/29866 discloses the cloning and sequencing of the *HP-2* gene, said gene being found to encode the tomato homolog of the *Arabidopsis* nuclear protein *DEETIOLATED1* (*DET1*). This publication further discloses that a point mutation and deletion mutation, both of which are located in exon 11 at the 3′ end of the coding sequence of *HP-2*, give rise to the previously-identified $hp\text{-}2^{j}$ and *hp-2* mutants respectively. In the case of the *hp-2* mutant, a point

mutation directs alternative splicing of intron 10 that leads to a nine base pair deletion in exon 11.

The *dg* mutant is phenotypically similar to other *hp* mutants, but has a much darker mature-green fruit, resulting from higher total chlorophyll content (Wann et al. 1985; Konsler 1973).Another significant difference between the phenotypes of the *dg* mutant and certain other photomorphogenic mutants such as the *hp-2*^{j} mutant is that red-ripe fruit of the *hp-2*^{j} mutants is sensitive to concentric cracking resulting in fruits with poor quality. Red-ripe fruit of the *dg* mutants are significantly more crack resistant and therefore of higher quality.

The map location of the *dg* mutation was unknown prior to the present invention.

It is a purpose of the present invention to provide an isolated nucleotide sequence that contains the mutation responsible for the *dg* photomorphogenic mutant of tomato plants.

It is a further purpose of the present invention to provide a polymorphic PCR-based DNA marker that may be used as a molecular diagnostic tool for the identification and selection of *dg* mutants.

It is a still further purpose of the present invention to provide a molecular diagnostic tool for use in the early selection of *dg* mutant plants at the seedling stage.

A yet further purpose of the present invention is to provide a molecular diagnostic tool that may be used for

genotypic selection in the production of photomorphogenic double mutants.

Another purpose of the invention as claimed herein is to provide a diagnostic tool that may be used to determine the presence of the *dg* mutation in a post-control setting in seed production.

A further purpose of the invention is to provide an efficient method for preparing double mutant plants for two photomorphogenic genes, wherein said method obviates the need for laborious test crosses.

Other purposes and advantages of the present invention will become apparent as the description proceeds.

Summary of the Invention

It has now been found that, despite the abovementioned phenotypic differences between the *dg* mutant and other photomorphogenic mutants, the mutation responsible for the *dark green (dg)* mutant is located within the same gene as the mutations responsible for both the *hp-2* and $hp\text{-}2^j$ mutants, namely the tomato homolog of the *DET1* gene (*HP-2*). Surprisingly, however, despite the fact that both of the known photomorphogenic mutations of the *DET1* gene (i.e. the $hp\text{-}2^j$ and *hp-2* mutations) are close to the 3′ end of the coding sequence, the *dg* mutation, which is disclosed for the first time herein, is located close to the 5′ end of the coding region of said gene.

The present invention relates to an isolated nucleotide sequence responsible for the tomato *dg* phenotype, wherein

said sequence comprises an altered tomato *DET1* gene sequence or fragment or homolog thereof, wherein the alteration in said altered sequence or fragment or homolog comprises an A-to-T transversion at nucleotide 29 of the second exon of said *DET1* gene sequence.

In one preferred embodiment of the invention, the isolated nucleotide sequence comprises the sequence shown in Fig. 2A.

The present invention is particularly directed to the use of the abovementioned isolated nucleotide sequence as a molecular diagnostic tool. Said tool may be used to aid the introgression of the *dg* mutation into various genetic backgrounds for the purpose of improving fruit quality and nutritional value.

Thus in one aspect, the present invention provides a method for detecting the presence of the *dg* mutation in a plant, comprising the steps of isolating the genomic DNA from said plant, amplifying a gene fragment containing said *dg* mutation from said genomic DNA by use of a PCR technique using primers designed to amplify the flanking regions of said *dg* mutation and detecting the presence of said *dg* mutation in said genomic DNA.

In one preferred embodiment of the above-disclosed method of the invention, the presence of the *dg* mutation is detected by the use of restriction site analysis, wherein the loss of an *Acl* I site indicates the presence of said mutation.

In another preferred embodiment of the method of the invention, the presence of the *dg* mutation is determined by direct sequencing of the amplified fragment and comparison of the sequence obtained with that shown in Fig. 2A.

In a further preferred embodiment of the method of the invention, the forward and reverse primers used to amplify the flanking regions of the *dg* mutation are:

5'-TTC TTC GGA TTG TCC ATG GT-3'
and
5'-CAC CAA TGC TAT GTG CCA AA-3',
respectively.

In another preferred embodiment, the plant in which the presence of the *dg* mutant is being detected is of the species *Lycopersicon esculentum*.

In another aspect, the invention also encompasses the use of the above-described method as a means of quality control, or post-control in seed production, for detecting the presence of the *dg* allele in cultivars and their parental lines. The term post-control is used herein to indicate quality control checks that are performed following seed production, in order to confirm the intended genotype of said seeds.

In yet another aspect, the invention is directed to a method for the determination of the presence of two different photomorphogenic mutations in a plant, wherein one of said mutations is the *dg* mutation, comprising detecting the presence of a photomorphogenic mutation other than the *dg* mutation by genotypic or phenotypic selection

means, and detecting the presence of the *dg* mutation by means of the method described hereinabove.

In one preferred embodiment of the immediately-preceding method, the phenotypic selection means for determining the presence of the non-*dg* mutation comprises germinating seeds obtained from the plant in which the presence of the mutations is being determined in a temperature controlled chamber, under a yellow plastic screen, said screen preventing transmittance of light having a wavelength less than 500nm, and selecting the non-etiolated seedlings eight days post-sowing.

The term non-etiolated refers to seedlings with significantly shorter hypocotyls and lower cotyledon mass, characterized by significantly higher anthocyanin and chlorophyll content. In other words: seedlings that appear as if they were grown in the presence of normal light levels, although in fact they were grown under suboptimal light conditions.

In another aspect, the present invention is also directed to a method for preparing double-mutant lines of *Lycopersicon esculentum* having genotype *dg/dg p/p*, wherein *p* represents any recessive photomorphogenic mutation that is genetically unlinked to the *dg* mutation, said method comprising the steps of:

a) cross-hybridization of a homozygous *dg/dg +/+* line (or plant) with a homozygous *+/+ p/p* line (or plant) to yield double heterozygous *dg/+ p/+* F_1 plants;
b) self-crossing of the F_1 plants obtained in step (a) in order to yield F_2 seeds;

c) light-sensitivity selection of light-hypersensitive seedlings germinated from F_2 seeds;

d) selection of light-hypersensitive seedlings containing the *dg/+ p/p* mutations, by use of the above-described method for detecting *dg/+* heterozygous plants;

e) self-crossing of the selected plants obtained in step (d) to generate F_3 seeds, and germination of said seeds under normal conditions;

f) selection of double homozygous *dg/dg p/p* seedlings from the seedlings obtained in step (e), by use of the above-described method for detecting the presence of the *dg* mutation in a plant.

The term "genetically unlinked" as used herein refers to the situation wherein the second photomorphogenic mutation is located within a gene other than *DET1*.

In one preferred embodiment of the immediately preceding method for preparing double-mutant lines, the recessive photomorphogenic mutation *p* is a mutation that causes photomorphogenic phenotypic effects similar or identical to those caused by the *hp-1* mutation.

In the present context the abovementioned "phenotypic effects similar or identical to those caused by the *hp-1* mutation" are those already described hereinabove, *i.e.*, exaggerated light responsiveness, higher anthocyanin levels, shorter hypocotyls, and greater fruit pigmentation (in comparison to their semi-isogenic wild type plants).

In a more preferred embodiment, the abovementioned recessive photomorphogenic mutation *p* that causes phenotypic effects similar or identical to those caused by

the *hp-1* mutation is a lycopene-enhancing mutation. In a particularly preferred embodiment, the lycopene-enhancing mutation is hp-1^{w}. The term "lycopene-enhancing mutation" in this context is used to describe photomorphogenic mutations that cause a significant increase in the amount of lycopene produced by the host plant containing said mutation, as compared to a plant bearing the wild-type, non-mutated gene.

In another preferred embodiment, the recessive photomorphogenic mutation *p* is the *hp-1* mutation.

The present invention is further directed to double-mutant hybrid plants of the species *Lycopersicon esculentum* having genotype *dg/dg p/p*, wherein *p* is as defined hereinabove. In one particularly preferred embodiment, said double-mutant hybrid plants have genotype *dg/dg hp-1/hp-1*. In another particularly preferred embodiment, the double-mutant hybrid plants have genotype *dg/dg* $hp\text{-}1^{w}/hp\text{-}1^{w}$.

According to a preferred embodiment of the invention, the abovementioned double-mutant hybrid plants are prepared by the method disclosed hereinabove.

All the above and other characteristics and advantages of the present invention will be further understood from the following illustrative and non-limitative examples of preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 Visual phenotypic characterization of heterozygous *hp-2^j/dg* F_1 plants. A. A photograph showing a typical *hp-2^j/dg* F_1 plant with mature-green fruits obtained from a cross between *hp-2^j* and *dg* mutants; B. A photograph showing developmental differences between heterozygous *hp-2^j/+* and *hp-2^j/dg* F_1 plants, two months after sowing; C. A photograph showing mature green and ripe-red fruits of heterozygous *hp-2^j/+* and 6 *hp-2^j/dg* F_1 plants.

Fig. 2 provides an alignment of nucleotide (A) and deduced amino acid sequence (B) of the first (I) and part of the second (II) exons of *DET1* gene in wild type (w.t.) and *dg* plants. The site of the mutation, in both the nucleotide and amino acid sequences, is indicated by an enlarged bold letter. The *Acl* I cleavage recognition sequence is underlined in the wild-type nucleotide sequence.

Fig. 3 demonstrates cleaved amplified polymorphic DNA marker that is used to identify *dg* mutant plants in cv Manapal background, in accordance with the method of the invention.

Fig. 4 depicts the segregation of the cleaved amplified polymorphic DNA marker for *dg* mutant plants in an F_2 population, showing the two homozygous genotypes (single bands) and the heterozygous genotype (two bands).

Detailed Description of Preferred Embodiments

In one of its aspects, as described hereinabove, the present invention provides a method for detecting the

presence of the *dg* mutation in a plant, comprising the steps of isolating the genomic DNA from said plant, amplifying a gene fragment containing said *dg* mutation from said genomic DNA by use of a PCR technique using primers designed to amplify the flanking regions of said *dg* mutation, digesting the PCR amplification product using the *Acl I* endonuclease, and detecting the presence of said *dg* mutation in said genomic DNA by means of electrophoresis in 1.0% agarose and staining with ethidium bromide.

The term "PCR" (or polymerase chain reaction) technique as used hereinabove and hereinbelow refers to a family of techniques that are based on the use of heat-stable polymerases for achieving the amplification (i.e. increase in number of copies) of specific DNA sequences by repeated polymerase reactions. This reaction can be used as a replacement for cloning: all that is required is knowledge of the nucleic acid sequence. In order to carry out PCR, primers are designed which are complementary to the sequence of interest. The primers are then generated by automated DNA synthesis.

PCR and other methods of amplifying DNA and/or RNA are well known in the art, and can be used according to the present invention without the need for undue experimentation, based on the teaching and guidance presented herein. Several PCR methods (as well as related techniques) are described, for example, in US patent Nos. 4,683,195, 4,683,202, 4,800,159, 4,965,188, as well as in Innis *et al.* eds., *PCR Protocols: A guide to method and applications*.

Examples

Methods

Plant material, crosses, growth conditions and experimental design

The *hp-2* and *hp-2*^{j} mutants and the corresponding semi-isogenic wild-type tomato seeds (Solanum *Lycopersicon esculentum* cv Money Maker) were kindly provided by R.E. Kendrick and M. Koornneef (Wageningen Agriculture University, the Netherlands). The *dg* mutant and its corresponding isogenic wild type tomato seeds (Solanum *Lycopersicon esculentum* cv Manapal) were kindly provided by R.T. Chetelat (Tomato Genetics Cooperative, UC Davis, USA). Seeds from wild-type tomato (Solanum *Lycopersicon esculentum* cv Ailsa Craig) and a line nearly isogenic and homozygous for the *hp-1* mutation were obtained from J.J Giovannoni (Texas A and M University, USA). Lines n474 and n935, which were used to sequence the *DET1* gene, are *dg* mutant lines developed by the late R. Frankel, D. Lapushner and I. Levin at the Volcani Center, Bet Dagan, Israel. Several wild-type tomato cultivars used in this study, such as: Ailsa-Craig, M82, VF-36, 189, 124, 139, NC8288 and Florida were from seed stocks available at the Volcani center.

The initial cross was carried out between *hp-2*^{j} and *dg* during the summer of 2000 in a screen-house at the Volcani Center, Bet Dagan, Israel. In this cross, the indeterminate *hp-2*^{j} (cv Money Maker) plants served as a maternal line, whereas a mixture of pollen derived from 10 unrelated determinate parental breeding lines, represented the paternal *dg* genotype. Seven of the F_1 plants resulting from the above cross were planted in an environmentally

controlled greenhouse at the Volcani Center during the winter of 2000/2001. All of these plants displayed a delayed growth development, and set mature-green and ripe-red fruits with a characteristic *dg* phenotype, identical to homozygous *dg/dg* plants (Figure 1). All of these F_1 plants were found to be true hybrids, using markers complementary to the *SELF-PRUNING* locus (Pnuelli et al. 1998). F_2 Seeds were individually extracted from each one of these plants at the end of the season.

Sixty-four F_2 seeds, from each one of the 7 original F_1, plants were germinated under a yellow plastic screen that prevents transmittance of light having a wavelength less than 500nm (Mochizuki and Kamimura, 1984), in the Spring of 2001 in an environmentally controlled greenhouse at a commercial nursery (Hish-Shtill, Nehalim, Israel). The photomorphogenic response of seedling hypocotyl length was visually compared to several wild-type genotypes germinated under the same conditions. Twenty-eight of the more etiolated F_2 seedlings, representing each one of the 7 original F_1 plants (A total of 196 plants), were planted in an open field at the Hula valley research station located in northern Israel.

DNA was extracted from 5 plants chosen at random from each one of the 7 F_2 populations (A total of 35 plants). These DNA samples were genotyped using a DNA marker developed based on a mutation discovered, in this research, in the tomato *DET1* homolog of *dg* mutants. Lycopene was extracted and its content determined in ripe-red fruits sampled from 21 of these genotyped plants. Fourteen plants that set an insufficient amount of fruits were discarded from further analysis.

F_3 Seeds were extracted individually from each one of the above F_2 plants. A sample of 20 F_3 seeds, derived from 6 heterozygous F_2 plants (*dg/hp-2^j*), representing 6 of the 7 original F_1 plants (A total of 120 seeds) were sampled. These seeds were germinated under yellow plastic screen in an environmentally controlled growth chamber (25°C day/18°C night) during the autumn of 2001. Hypocotyl-length of the resulting seedlings was individually measured after 7 days, and compared to the length of wild type seedlings.

A linkage analysis study between the tomato *DET1* locus and the aggregated photomorphogenic response characterizing *dg* mutants was carried out using F_2 seeds of a cross between determinate *dg* mutant plants and wild type plants (cv PETO4303). These seeds were allowed to germinate under yellow plastic screen in an environmentally controlled growth chamber during the autumn of 2001 (25°C day/18°C night). Hypocotyl-length of individual F_2 seedlings was measured 9 days after sowing and their genotype determined using the DNA marker developed in this study.

Genomic DNA extraction and cDNA preparation

Genomic DNA was extracted from individual plants. The extraction procedure was according to Fulton *et al.* 1995.

PCR primers

Sequence analysis and locus-specific primer design were carried out with the DNAMAN, Sequence Analysis Software version 4.1 (Lynnon BioSoft, Quebec, Canada). All DNA primers used during the course of this study were purchased from M.B.C Molecular Biology Center Ltd., Ness-Ziona,

Israel. PCR primers used to clone overlapping fragments spanning the entire coding sequence of the tomato *DET1* gene were:

TDR1: 5'-GTA CAC CTT AGT TGC TCG AGG GCG TG-3'
CRISR: 5'-GTG ATT TCT AGG TTG ATT TCA ATC TAG AG-3'
TDF2: 5'-GAT CCT AAT TCG AGC CCT CCT T-3'
TDF1: 5'-GGA TGG AGC TAT ACT TGA CGA AAG GG-3'
TDR: 5'-GCC GTT GCT TTA TAC CGC TCA GGA AA-3'
TDR2: 5'-CAC TAG CAT CTA CGG GTC TGT TAT G-3'

PCR primers used to amplify the tomato *DET1* genomic DNA fragment flanking the *dg* mutation locus were:

TD-M2 F1: 5'-TTC TTC GGA TTG TCC ATG GT-3'
TD-M2 R1: 5'-CAC CAA TGC TAT GTG CCA AA-3'

The abovementioned TD-M2 F1 primer anneals to the first intron of the tomato *DET1* sequence (bases 2171-2190 in NCBI accession AJ224356), while the TD-M2 R1 primer anneals to exon 3 of the tomato *DET1* sequence (bases 2848-2867 in NCBI accession AJ224356).

PCR reaction

The amplification reactions (25 μl final volume) were performed with 10 ng template DNA, 25 mM TAPS (pH=9.3 at 25°C), 50 mM KCl, 2mM $MgCl_2$, 1mM β-mercaptoethanol, 0.2 mM of each of the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 10 ng of each of two primers and 1 unit of thermostable *Taq* DNA polymerase (SuperNova *Taq* polymerase, Madi Ltd., Rishon Le Zion, Israel). Reactions were carried out in an automated thermocycler (MJ Research Inc., Watertown, MA, USA). Initial incubation was

at 94°C for 1 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1.5 min. Final polymerization at 72°C was carried out, for 3 min, after the above cycles have been completed. The PCR amplification products were visualized by electrophoresis in 1.0% agarose gels and detected by staining with ethidium bromide.

Cloning and sequencing of the tomato DET1 gene from dg mutant plants

Total RNA was extracted from breaker fruits (200 mg fresh weight) of individual *dg* mutant plants representing 2 independent lycopene-rich tomato breeding lines, n474 and n935 and the open pollinated wild-type cultivar Ailsa-Craig. The RNA extraction was carried out using the TRIzol reagent system (GibcoBRL Life Technologies, Gaithersburg, MD, USA). The total RNA was used as template for first strand cDNA synthesis using the Superscript pre-amplification system (GibcoBRL Life Technologies, U.K.). The cDNA prepared was used as template, in a PCR reaction, to amplify three overlapping fragments of the gene encoding the tomato *DET1* from the two *dg* mutant plants. A fragment, 1783 bp in length, was amplified in two sequential PCR reactions. The first reaction was primed with TDF2-TDR2 primer combination. The resulting DNA product, 1816 bp in length, was excised from an agarose gel, purified using the GENECLEAN II kit (BIO 101 Inc., La Jolla CA, USA), and used for a nested PCR reaction primed with TDF2-CRISR. The two other fragments were amplified directly from the cDNA template using the primer combinations TDF2-TDR and TDF1-CRISR. The three PCR products were then cloned into pGEM-T Easy vector using the pGEM-T Easy Vector Systems according

to the manufacturer recommendations (Promega Corporation, Madison, WI, USA). Three independent clones of each of the three amplified fragments were sequenced, based on the vector T7, SP6, and the tomato *DET1* complementary primers (TDF2, TDF1 and TDR1), using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Equivalent fragments were cloned and sequenced from the wild type cv Ailsa Craig.

Lycopene extraction and analytical determination

Lycopene was extracted from pericarp tissue of fresh ripe-red fruits. Pericarp tissue of a sample of 20 fruits per individual plant was minced to puree in a blender. An aliquot of 2 g puree was taken from each sample and stirred with 40 ml extraction buffer for 30 min in the dark. The extraction buffer consisted of n-hexane:isopropanol:acetone (2:1:1) and contained 0.05% BHT. Phase separation was achieved by the addition of 20 ml 0.1M NaCl. The organic upper-phase was collected for analysis. Spectrophotometric absorbance at 472 nm was used to calculate lycopene concentration using E1%=3450 (Davis, 1965).

Statistical analyses

Statistical analyses were carried out with the JMP Statistical Discovery software (SAS Institute Inc., Cary, NC, USA).

Example 1

Allele test of *dg* and *hp-2^j* mutants

Seven F_1 hybrid *hp-2^j/dg* plants initially indicated that *dg* and *hp-2^j* are allelic. All of these F_1 plants set fruit that turn extremely green at the mature-green fruit developmental stage (Fig. 1). Such extreme greening is a characteristic phenotype of *dg* tomato mutants and appears much darker than *hp-2^j* mutants (not shown). These seven plants were confirmed as true hybrids using a PCR genotyping protocol based on tomato *SELF-PRUNING* (*SP*) gene sequence (Pnueli et al. 1998). All 7 F_1 plants were found to be heterozygous at the *SP* locus as would be expected in this cross where an indeterminate *hp-2^j/hp-2^j* maternal line was crossed with a determinate *dg/dg* paternal line.

To extend the analysis to F_2 generation, seeds were individually extracted from each of the 7 hybrid plants and a sample of 64 seeds, from each plant, was allowed to germinate under yellow plastic screen that eliminates spectral transmittance of light wave-length below 500 nm. Although variable, all of these F_2 seedlings displayed a divergent hypersensitive photomorphogenic phenotype, visualized as much shorter hypocotyl lengths, in comparison to wild type lines germinated under the same conditions (i.e. cv Money Maker and M82; data not shown).

To validate the allelic relationship between *dg* and *hp-2^j*, adult F_2 plants were also analyzed. Twenty-eight of the more etiolated F_2 seedlings, representing each one of the 7 original F_1 plants (A total of 196 plants), were planted in an open field. Five plants from each of these 7 F_2 populations were chosen at random for genotyping and fruit

lycopene analysis (a total of 35 plants). Genotyping the *DET1* locus in these plants (described hereinbelow) was carried out using DNA samples extracted from true leaves of each individual plant. Red-ripe fruits of 21 plants were analyzed for lycopene content. The fruit lycopene content of these F_2 plants exceeded those of their wild type controls (Table 1). No $hp\text{-}2^j/hp\text{-}2^j$ genotype was found among the plants that participated in this analysis, because of prior selection of the more etiolated plants for further analysis (see above). This genotype most probably failed to enter this selection because of the extremely slow seedling development, short hypocotyls and the severe deetiolated phenotypes it usually displays (Table 2).

Table 1. Lycopene content in mature red fruit of parental lines and randomly chosen F_2 plants. Different superscript letters represent statistically significant differences between genotypic means ($P<0.05$), based on Tukey-Kramer HSD test (Kramer, 1956).

Genotype	Line	N	Lycopene±S.E. (ppm)	Lycopene range (ppm)
+/+	M82	6	$72^B \pm 2$	64-78
+/+	Money maker	2	$83^B \pm 5$	79-88
$hp\text{-}2^j/hp\text{-}2^j$	Money maker	2	$171^A \pm 25$	146-197
dg/dg	LRT915	1	245^A	-
dg/dg	F_2	6	$224^A \pm 13$	182-263
$hp\text{-}2^j/dg$	F_2	15	$193^A \pm 10$	121-262

Table 2. Hypocotyl-length photomorphogenic response of tomato wild-type and mutant seedlings at the *DET1* and *HP-1* loci. Seedlings were grown under yellow plastic screen for 7 days after sowing. Different superscript letters represent statistically significant differences between means (P<0.05), within each genetic background, based on the Tukey-Kramer HSD test (Kramer, 1956).

Genotype	Genetic background	N	Hypocotyl length (mean±S.E.)
+/+	Money maker	39	6.4^{A} ±0.2
hp-2/hp-2	Money maker	38	5.7^{B} ±0.1
hp-2^j/hp-2^j	Money maker	14	2.5^{C} ±0.3
+/+	Ailsa Craig	40	7.5^{A} ±0.1
hp-1/hp-1	Ailsa Craig	40	4.7^{B} ±0.1
+/+	M82	73	7.5^{A} ±0.1
hp-1/hp-1	M82	40	5.0^{B} ±0.1
dg/dg	M82	40	4.9^{B} ±0.2

The allelic test between *dg* and *hp-2^j* was completed by hypocotyl-length photomorphogenic response analysis of F_3 seedlings. F_3 seeds representing heterozygous *hp-2^j/dg* plants from 6 of the 7 F_2 populations were collected separately and were germinated in a temperature controlled growth chamber under yellow plastic screen. Hypocotyl length of single plants was measured 7 days after sowing. Results demonstrate that all of the segregating F_3 *hp-2^j/dg* seedlings were hypersensitive-photomorphogenic as compared to the wild type controls (Table 3).

Table 3. Hypocotyl-length photomorphogenic response of wild type and segregating F_3 *hp-2^j/dg* seedlings. Seedlings were grown under yellow plastic screen for 7 days after sowing. Different superscript letters represent statistically significant differences between means (P<0.05) based on Tukey-Kramer HSD test (Kramer, 1956).

Genotype	N	line	Hypocotyl length (cm) Mean± S.E	Range
+/+	18	Money maker	$5.6^{A}\pm0.1$	4.5-6.6
Segregating *hp-2^j/dg*	62	F_3	$2.8^{B}\pm0.1$	1.2-4.4

Example 2

Allelic interaction between the *dg* and *hp-2^j* alleles of the *DET1* gene significantly increases fruit lycopene content

A homozygous paternal *dg* line, selected for by the primer combination presented herein, was crossed with 3 isogenic homozygous maternal lines harboring the wild-type *DET1* allele (+) and the mutant alleles: *hp-2* and the *hp-2^j*. The parental lines and the F_1 hybrids were planted in randomized block design- 3 blocks, each containing a plot of 10 plants of each of the parental and F_1 hybrids. Ripe-red tomato fruits were harvested from each plot, weighted and analyzed for lycopene content. Results show that the mutant hybrid *dg/hp-2^j* produces, on average, a statistically significant higher level of lycopene which exceeds those of the *dg/dg* and *hp-2^j/hp-2^j* genotypes by 25.8 and 37.6 %, respectively (Table 4). These effects remained statistically significant after adjusting for fruit weight: slightly decreased compared to the *hp-2^j/hp-2^j* genotype (35.7%), but significantly increased compared to the *dg/dg* genotype (37.1%). These results show that specifically combining the *dg* and the *hp-2^j* alleles of the *DET1* gene can significantly increase lycopene concentration in the red-ripe tomato fruit and that this increase remains statistically significant after adjusting for fruit weight.

Table 4. Weight adjusted and non adjusted average lycopene concentrations in wild-type, *DET1* mutant lines and their F_1 hybrids. Different superscript letters represent statistically significant differences between means ($P<0.05$) based on Tukey-Kramer HSD test (Kramer, 1956).

Genotype	Genetic background	Lycopene (ppm)	
		Non adjusted	Weight adjusted
+/+	Money maker	83^D	72^D
hp-2/hp-2	Money maker	151^C	135^C
$hp\text{-}2^j/hp\text{-}2^j$	Money maker	182^B	207^B
dg/dg	M82	198^B	205^B
dg/+	F_1	103^D	123^C
dg/hp-2	F_1	202^B	212^B
$dg/hp\text{-}2^j$	F_1	249^A	281^A

Example 3

Sequence characterization of the tomato *DET1* in *dg* mutants

Two independently developed lycopene-rich breeding lines, homozygous for the *dg* mutation, were used for sequence analysis. The entire coding region of the tomato *DET1* cDNA was thoroughly sequenced. A single transversion of Thymine-to-Adenine was observed when *dg* mutant sequence was compared to wild type. This single base transversion was found to be located at nucleotide 29 of the second exon of the *DET1* gene sequence (Fig. 2A), and thus its deduced amino-acid substitution would be Asparagine^{34} to Isoleucine^{34} (Fig. 2B). This mutation also renders the *dg* mutant allele resistant to cleavage by the *Acl* I restriction endonuclease. This enzyme (whose recognition sequence is AACGTT, indicated by underlining in the nucleotide sequence given in Fig. 2A) cleaves the wild-type

sequence into two fragments of size 578 and 119 bp respectively.

Example 4

Diagnostic tool for identifying the *dg* mutation

A DNA marker for use as a molecular diagnostic tool for identifying *dg* mutant plants based on the sequence results (Fig. 2) was developed. The PCR primers (TD-M2 F1 and TD-M2 R1, sequence given hereinabove) were designed to amplify the genomic DNA sequences flanking the *dg* mutation. These genomic sequences were amplified, incubated with *Acl* I (recognition sequence: AACGTT, indicated by underlining in the nucleotide sequence shown in Fig. 2A) and a clear polymorphism between *dg* and several wild-type plants representing 9 lines or cultivars (Money Maker, Ailsa Craig, M82, VF-36, 189, 124, 139, NC8288 and Florida; Data not shown) was seen. In the case of the homozygous *dg* mutant plants, a single amplified band of 679 bp was seen, while in wild-type plants, cleavage by *Acl I* led to the appearance of one 578 bp fragment and one 119 bp fragment. As expected, plants heterozygous for the *dg* mutation yielded the following three bands following *Acl I* cleavage: 679 bp, 578 bp and 119 bp. In order to substantiate these results, the *DET1* locus in *dg* mutant plants of the Manapal cultivar was genotyped and compared to that of the fully isogenic wild type plants. The polymorphism obtained (Fig. 3), confirms that *dg* is a mutation at the tomato homolog of *DET1* gene. The segregation of individual plants using the DNA marker is presented in Fig 4.

Example 5

Linkage analysis between the *DET1* locus and the photomorphogenic response

A linkage analysis study was carried out to test the association between the *DET1* locus and the characteristic hypersensitive-photomorphogenic response displayed by *dg* mutant plants (i.e. hypocotyl-length phenotype). For this purpose, F_2 seeds of a cross between determinate *dg* mutant plants and wild type plants (cultivar PETO4303) were germinated under a yellow plastic screen in a controlled growth chamber. Nine days after sowing, the hypocotyl-lengths of individual seedlings were recorded and their *DET1* locus genotyped using the DNA marker described above. The results demonstrate a clear association between the *DET1* locus and hypocotyl-length (Table 5). Homozygous recessive *dg/dg* seedlings displayed a highly statistically significant shorter average hypocotyl-length, indicative of a more hypersensitive-photomorphogenic response, in comparison to the two other genotypic groups (12<LOD Score<13, R^2=51.1%). These results confirm that the mutation identified in the *DET1* locus of *dg* mutant plants is associated with one of its main characteristic phenotypes, i.e. shorter hypocotyl-length of seedlings.

Table 5. Linkage analysis between the tomato *DET1* locus and the hypocotyl-length photomorphogenic response. Seedlings were grown under yellow plastic screen for 9 days after sowing. Different superscript letters represent statistically significant differences between means ($P<0.05$) based on Tukey-Kramer HSD test (Kramer, 1956).

Genotype	N	Hypocotyl length ± S.E. (cm)	LOD score	R^2
+/+	18	$7.7^A \pm 0.3$	**12<LOD<13**	**51.1%**
dg/+	35	$7.7^A \pm 0.2$		
dg/dg	33	$5.4^B \pm 0.2$		

Example 6

Incorporation of two genetically unlinked lycopene enhancing mutations in a single tomato hybrid: *Experimental Approach*

A common practice among breeders is to combine or incorporate two or more mutations positively affecting the same trait. Such procedure can be verified by laborious and time consuming test crosses. The diagnostic tool produced herein can facilitate the incorporation of two light hypersensitive lycopene-enhancing mutations in a single plant or breeding line.

Several mutations in tomato positively affect lycopene content in the mature tomato fruit. Of these, at least 5 show a significant hypersensitive light response. These include:

1. *High pigment-1* (*hp-1*)
2. *High pigment-1*^{w} (*hp-1*^{w})
3. *High pigment-2* (*hp-2*)

4. *High pigment-2^j* (*hp-2^j*)
5. *Dark green* (*dg*)

The *hp-1* and *hp-1^w* mutations map to the *HP-1* locus on the tomato chromosome 2 (Yen et al., 1997). The *hp-2*, *hp-2^j* and, in accordance with the present invention, *dg* mutations map to the *HP-2* locus on the tomato chromosome 1 (Mustilli et. al, 1999). Incorporation of lycopene enhancing *dg* and either one of the two mutations that map to the *HP-1* locus (*hp-1* and *hp-1^w*) can be more efficiently achieved through the following procedure:

1. Cross homozygous *dg* with homozygous *hp-1* mutants to generate double heterozygous F_1 plants:

$$dg/dg \; +/+ \; X \; +/+ \; hp\text{-}1/hp\text{-}1$$

$$\downarrow$$

$$dg/+ \; hp\text{-}1/+$$

2. Self-cross the F_1 double heterozygous plants to generate F_2 seeds. These F_2 seeds will segregate into 9 genotypes that can be further categorized into two groups according to their light hypersensitivity (Table 6).

3. Germinate the F_2 seeds in a temperature controlled chamber, under yellow plastic screen that prevents transmittance of light having a wavelength of less than 500nm (Mochizuki and Kamimura, 1984). Discard the etiolated seedlings representing the non-hypersensitive genotypes eight days post-sowing (see Table 6).

4. Use the diagnostic tool (see Example 4 hereinabove) on DNA extracted from the remaining, light hypersensitive seedlings, in order to detect the presence of the *dg* mutation. Select heterozygous *dg+* seedlings that comprise a single genotypic group of plants that are already *hp-1* homozygous (see Table 6).

5. Self-cross the selected F_2 *dg/+ hp-1/hp-1* plants to generate F_3 seeds. These seeds will segregate into 3 genotypic groups: *dg/dg hp-1/hp-1*, 2. *dg/+ hp-1/hp-1*, and 3. *+/+ hp-1/hp-1*.

6. Germinate the F_3 seeds under normal conditions.

7. Use the diagnostic tool (Example 4, hereinabove) on DNA extracted from the F_3 seedlings. Select homozygous *dg/dg* plants. These plants will necessarily be double mutant plants, combining two lycopene-enhancing mutations.

Table 6

Light hypersensitive	
Yes	no
dg/dg hp-1/hp-1	*dg/+ hp-1/+*
dg/dg hp-1/+	*dg/+ +/+*
dg/dg +/+	++ hp-1+
dg/+ hp-1/hp-1	+/+ +/+
+/+ hp1/hp1	

Example 7

Incorporation of two genetically unlinked lycopene enhancing mutations in a single tomato hybrid significantly increases lycopene yield: *Working Example*

Two semi-isogenic hybrids, one homozygous for the *hp-1* mutation, *hp-1/hp-1*, and the other for the *dg* mutation, *dg/dg*, were crossed hybridized to yield F_1 plants (*hp-1/+ dg/+*). These F_1 plants were self-hybridized to yield F_2 seedlings. These F_2 seedlings were screened using a yellow plastic screen, genotyped and self-hybridized to yield F_3 plants, as outlined in Example 6. These F_3 plants were genotyped again using the primer combination presented herein and self-hybridized to yield F_4 plants as also outlined in Example 6. These plants are, as outlined in Example 6, double mutant plants (*hp-1/hp-1 dg/dg*). Two horticulturally acceptable plants were selected and allowed to self hybridize to yield two F_5 lines. These F_5 lines were cross hybridized to yield a double mutant hybrid. This hybrid was tested, together with the semi-isogenic single mutant hybrids used in the initial cross (see above), in 4

locations in northern Israel during the spring season under open field conditions. Results presented in Table 7 show that, unexpectedly, the lycopene yield of the double mutant hybrid is statistically higher compared to its isogenic single mutant hybrids. The increase in lycopene yield of the double mutant hybrid was 19 and 61% compared to the lycopene yield of the *dg/dg* and *hp-1/hp-1* single mutant hybrids, respectively.

Table 7. Lycopene yield of single and double mutant hybrid cultivars carrying hypersensitive lycopene-enhancing mutations. Different superscript letters represent statistically significant differences between means (P<0.05) based on Tukey-Kramer HSD test (Kramer, 1956).

Cultivar genotype	Lycopene yield (gr/dunam*)
+/+ hp-1/hp-1	1136^{C}
dg/dg +/+	1538^{B}
dg/dg hp-1/hp-1	1824^{A}

* 1 dunam= 1000 square meters

Example 8

Use of the diagnostic tool for post control analysis of parental lines and hybrid seeds.

Seed companies often use a battery of molecular markers for post- or quality- control of parental seed stocks and hybrid-seeds. Several commercial lycopene-rich tomato cultivars carry the *dg* mutation either at a homozygous or heterozygous state. Up until now, detection of the *dg* trait within a particular stock could only be performed by the lengthy procedure of germinating samples of the seeds, and

performing complicated phenotypic analyses on the parental cultivars and subsequent generations.

The diagnostic tool developed in this study (see Example 3, hereinabove) can be used to positively detect the *dg* allele in such cultivars and their parental lines, and thus enable post-production quality control to be carried out over a time scale of 1-2 days instead of weeks or months.

References

Chory J (1993) Out of darkness: mutants reveal pathways controlling light-regulated development in plants. Trends in Genet 9:167-172

Davis BH (1965) Analysis of carotenoid pigments. In: Chemistry and Biochemistry of Plant Pigments. Goodwin TW ed. Academic Press. London and New York p.530.

Fulton TM, Chunwongse J, Tansley SD (1995) Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol Rep 13:207-209

Kerckhoff LHJ, Kendrick RE (1997) Photocontrol of antocyanin biosynthesis in tomato. J Plant Res 110:141-149

Konsler TR (1973) Three mutants appearing in 'Manapal' tomato. HortSci 8:331-333

Kramer CY (1956) Extension of multiple range tests to group means with unequal number of replications. Biometrics 12: 309-310

Mochizuki T, and Kamimura S (1984) Inheritance of vitamin C content and its relation to other characters in crosses between hp and og varieties of tomatoes. Eucarpia Tomato working group. Synopsis IX meeting 22-24 May, Wageningen, The Netherlands, pp 8-13

Mustilli AC, Fenzi F, Ciliento R, Alfano F Bowler C (1999) Phenotype of the tomato *high pigment-2* is caused by a mutation in the tomato homolog of *DEETIOLATED1*. Plant Cell 11:145-157

Pnuelli L, Carmel-Goren L, Hareven D, Gutfinger T, Alvarez J, Ganal M, Zamir D, and Lifschitz E (1998) The *SELF-PRUNING* gene of tomato regulates the reproductive switching of sympodial meristems and is the ortholog of *CEN* and *TFL1*. Development 125:1979-1989

Reynard GB (1956) Origin of Webb Special (Black Queen) in tomato. Rep Tomato Genet Coop 40: 44-64

Soressi GP (1975) New spontaneous or chemically-induced fruit ripening tomato mutants. Rep. Tomato Genet Coop 25:21-22

Van Tuinen A, Cordonnier-Prat M-M, Pratt LH, Verkerk R, Zabel P, and Koornneef M (1997). The mapping of phytochrome genes and photomorphogenic mutants of tomato. Theor Appl Genet 94:115-122

Wann EV, Jourdain EL, Pressey R Lyon BG (1985) Effect of mutant genotypes *hp* og^c and *dg* og^c on tomato fruit quality. J Amer Hort Sci 110:212-215

Wann, E.V. (1997) Tomato germplasm lines T4065, T4099, T5019, and T5020 with unique genotypes that enhance fruit quality. HortSci 32: 747-748

Yen H, Shelton A, Howard L, Vrebalov J Giovanonni J (1997) The tomato high-pigment (*hp*) locus maps to chromosome 2 and influences plastome copy number and fruit quality. Theor And Appl Genet 95: 1069-1079.

Claims

1. An isolated nucleotide sequence responsible for the tomato *dark green* (*dg*) phenotype, wherein said sequence comprises an altered tomato *DET1* gene sequence or fragment thereof, wherein said the alteration in said altered sequence or fragment comprises an A-to-T transversion at nucleotide 29 of the second exon of said *DET1* gene sequence.

2. The isolated nucleotide sequence according to claim 1, wherein said sequence comprises the sequence shown in Fig. 2A.

3. A method for detecting the presence of the *dg* mutation in a plant, comprising the steps of isolating the genomic DNA from said plant, amplifying a gene fragment containing said *dg* mutation from said genomic DNA by use of a PCR technique and determining the presence of said *dg* mutation in said genomic DNA.

4. The method according to claim 3, wherein the presence of the *dg* mutation is determined by the use of restriction site analysis, wherein the loss of an *Acl I* site indicates the presence of said mutation.

5. The method according to claim 3, wherein the presence of the *dg* mutation is determined by direct sequencing of the amplified fragment and comparison of the sequence obtained with that shown in Fig. 2A.

6. The method according to claim 3, wherein the plant in which the presence of the *dg* mutant is being detected is of the species *Lycopersicon esculentum*.

7. Use of the method according to claim 3 as a means of post-control in seed production.

8. A method for the determination of the presence of two different photomorphogenic mutations in a plant, wherein one of said mutations is the *dg* mutation, comprising detecting the presence of a photomorphogenic mutation other than the *dg* mutation by either genotypic or phenotypic selection means, and detecting the presence of the *dg* mutation by means of the method according to claim 3.

9. The method according to claim 8, wherein the phenotypic selection means for determining the presence of the non-*dg* photomorphogenic mutation comprises germinating seeds obtained from the plant in which the presence of the mutations is being determined in a temperature controlled chamber, under a yellow plastic screen that is opaque to light having a wavelength less than 500nm, and selecting non-etiolated seedlings.

10. A method for preparing double-mutant lines of *Lycopersicon esculentum* having genotype *dg/dg p/p*, wherein *p* represents any recessive photomorphogenic mutation that is genetically unlinked to the *dg* mutation, said method comprising the steps of:

a) cross-hybridization of a homozygous *dg/dg* line or plant with a homozygous *p/p* line or plant to yield double heterozygous *dg/+ p/+* F_1 plants;
b) self-crossing of the F_1 plants obtained in step (a) in order to yield F_2 seeds;

c) light-sensitivity selection of light-hypersensitive seedlings germinated from F_2 seeds;
d) selection of light-hypersensitive seedlings containing the *dg/+ p/p* mutations, by use of the method of claim 3;
e) self-crossing of the selected plants obtained in step (d) to generate F_3 seeds, and germination of said seeds under normal conditions;
f) selection of double homozygous *dg/dg p/p* seedlings from the seedlings obtained in step (e), by use of the method of claim 3.

11. A method according to claim 10, wherein recessive photomorphogenic mutation *p* is a mutation that causes photomorphogenic phenotypic effects similar or identical to those caused by the *hp-1* mutation.

12. A method according to claim 11, wherein the recessive photomorphogenic mutation *p* is a lycopene-enhancing mutation.

13. A method according to claim 12, wherein the lycopene-enhancing mutation is *hp-1*^{w}.

14. A method according to claim 11, wherein *p* is the *hp-1* mutation.

15. Double-mutant hybrid plants of the species *Lycopersicon esculentum* having genotype *dg/dg p/p*.

16. Double-mutant hybrid plants according to claim 15, wherein said plants have genotype *dg/dg hp-1/hp-1*.

17. Double-mutant hybrid plants according to claim 15, wherein said plants have genotype *dg/dg* $hp\text{-}1^{w}/hp\text{-}1^{w}$.

Figure 1

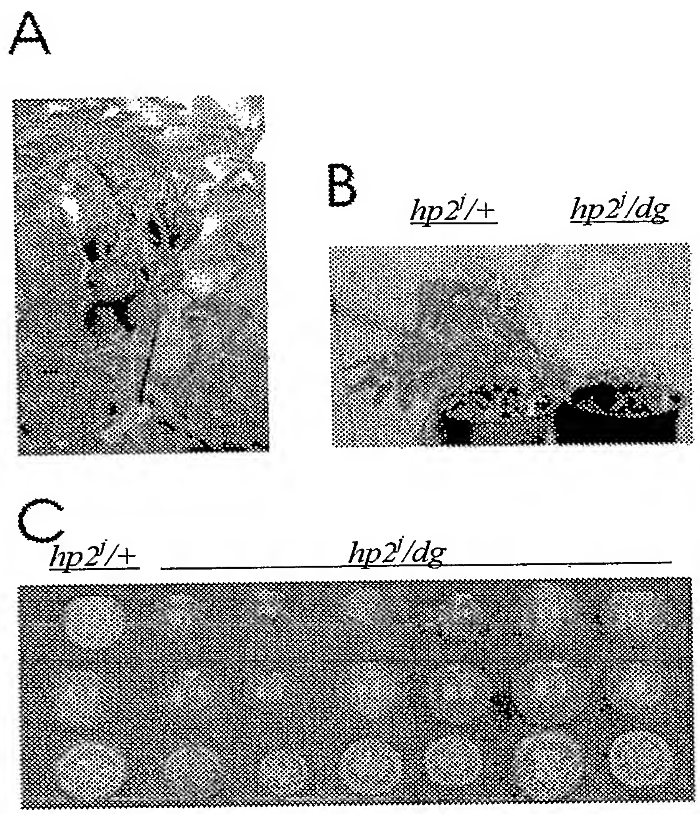

Figure 2

A. Nucleotide

```
w.t. I. ATGTTCAAAACTAACAATGTTACCGCCAGGCTTTTTGAGCGCCAGATTTGCACCCCTGCTCC
dg   I. ATGTTCAAAACTAACAATGTTACCGCCAGGCTTTTTGAGCGCCAGATTTGCACCCCTGCTCC
        **************************************************************

w.t. TGGCACCAGC II. ATCCATCGTGCCAGAAGATTTTATGAGAACGTTGTACCAAGTTATACCAT
dg   TGGCACCAGC II. ATCCATCGTGCCAGAAGATTTTATGAGATCGTTGTACCAAGTTATACCAT
     **********     **************************** *********************

w.t. ATACGATGTTGAATGTCCCGACCATTCATTTCGCAAGTTCACGGATGACGGT
dg   ATACGATGTTGAATGTCCCGACCATTCATTTCGCAAGTTCACGGATGACGGT
     ****************************************************
```

B. Amino Acid

```
w.t. I. MFKTNNVTARLFERQICTPAPGTS II. IHRARRFYENVVPSYTIYDVECPDHSFRKFTDDG
dg   I. MFKTNNVTARLFERQICTPAPGTS II. IHRARRFYEIVVPSYTIYDVECPDHSFRKFTDDG
        ************************     ********* ************************
```

Figure 3

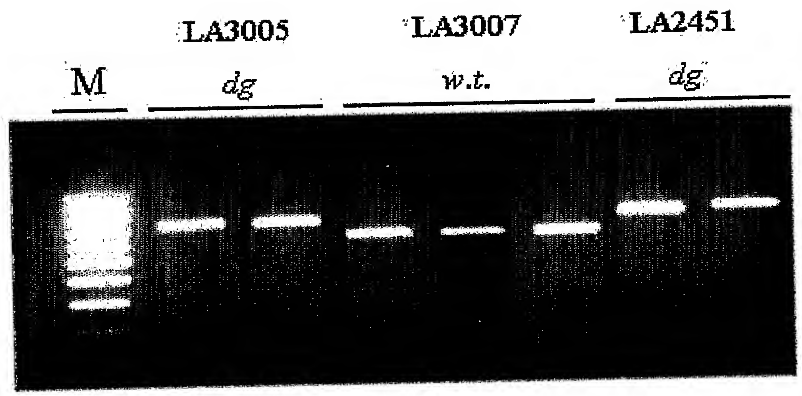

Figure 4